PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/12, A01K 67/027, C07K 14/745 | A1

(11) International Publication Number: **WO 98/15627**

(43) International Publication Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/GB97/02806

(22) International Filing Date: 10 October 1997 (10.10.97)

(30) Priority Data:
9621113.1 10 October 1996 (10.10.96) GB

(71) Applicants *(for all designated States except US)*: UNIVERSITY OF SOUTHAMPTON [GB/GB]; Highfield, Southampton SO17 1BJ (GB). ROYAL FREE HOSPITAL SCHOOL OF MEDICINE [GB/GB]; Rowland Hill Street, London NW3 2PF (GB).

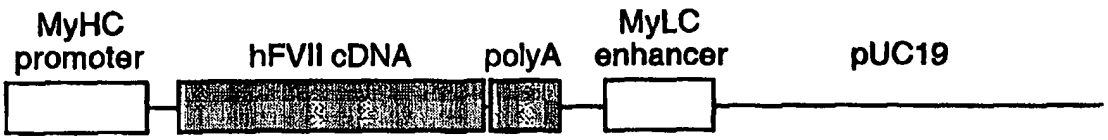
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MACLEAN, Norman [GB/GB]; 10 Russell Place, Highfield, Southampton SO17 1NU (GB). WILLIAMS, Darren, William [GB/GB]; 51 Ridgeway Road, Rumney, Cardiff CF3 9AB (GB). GOLDSPINK, Geoffrey [GB/GB]; Brambledene, East Common, Harpenden, Hertfordshire AL5 1DQ (GB). MÜLLER, Ferenc [HU/GB]; 51 Ridgeway Road, Rumney, Cardiff CF3 9AB (GB).

(74) Agent: WOODS, Geoffrey, Corlett; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PRODUCTION OF RECOMBINANT PEPTIDE BY TRANSGENIC FISH



(57) Abstract

The invention provides an expression construct for expressing a polypeptide in fish, which construct comprises a nucleic acid coding sequence encoding the polypeptide operably linked to a regulatory sequence capable of directing the expression of the coding sequence in a cell of the fish. It also provides methods of using such constructs to produce recombinant polypeptides in transgenic fish.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

PRODUCTION OF RECOMBINANT PEPTIDE BY TRANSGENIC FISH

Field of the Invention

This invention relates to the expression of nucleic acid sequences, typically heterologous sequences, encoding pharmaceutically or biologically active proteins, in fish. This generates recombinant polypeptides which can then be harvested from the fish or their eggs.

Background to the Invention

Certain systems exist for the expression of heterologous recombinant proteins. These include microbial systems, such as yeast and *E. coli*, and animal systems of two types, tissue culture and whole animal systems.

Microbial systems suffer from the drawback that they often fail to glycosylate or fold the heterologous protein correctly. In many cases, this means that the protein is biologically inactive, especially when an eukaryotic protein is produced in a prokaryotic system like *E. coli* where no glycosylation occurs.

Cell cultures, typically mammalian cell cultures, are an alternative as they are capable of effecting folding and glycosylation. However, while cell culture involves relatively simple experimental procedures and produces rapid reproducible results, it is extremely expensive; using recombinant Factor VIII prepared Chinese Hamster Ovary (CHO) cells, it costs around £80,000 per year to treat a haemophilia patient.

A further alternative is the introduction of cloned sequences into whole animal systems *in vivo*, which should maximise the possibility of correct expression of the gene construct since the introduced sequences are exposed to a complete spectrum of cell-specific signals. For example, a less expensive way to produce heterologous proteins than using cell cultures is to produce the protein in the milk of transgenic cows or sheep. Serious problems remain, however. The lytic enzymes contained in the milk hydrolyse the heterologous proteins unless they are highly resistant to degradation. Further, large mammals such as cows and sheep require extensive care. Also, as some diseases are common to non-human mammals and humans, there may be a danger that recombinant products produced in mammals will transmit pathogenic viruses and/or prions to human patients.

Recently, fish have been used to express heterologous proteins and a number of workers have produced stable transgenic fish lines which express a transgene in the F1 generation: these include lines expressing genes which encode, amongst others, growth hormone and antifreeze protein, as well as bacterial reporter genes such as CAT and LacZ.

Fish have a number of advantages for expression of recombinant proteins: (i) they are cheap to produce in large numbers; (ii) technology is available for the production of large numbers of transgenic fish; and (iii) as no known viruses or prions infect both humans and fish, transmission of disease is highly unlikely.

Summary of the Invention

The present inventors have found that, using a construct comprising DNA encoding the human Factor VII protein under the control of the CMV promoter, it is possible to express Factor VII transiently in transgenic zebrafish embryos. Furthermore, the recombinant Factor VII protein produced in these embryos is biologically active. Thus, it has been demonstrated that transgenic fish can be used to produce biologically active pharmaceutical polypeptides, especially in their embryonic tissues.

The inventors have also characterised the 5' region of the gene for a new member of the carp myosin heavy chain (MyoHC) gene family, the FG2 myosin heavy chain gene (Gauvry *et al.* (1996) Eur. J. Biochem, Vol 236; 887-894, incorporated herein by reference). Sequencing of the 5' region has revealed the presence of TATA and CCAAAT boxes typical of a promoter region. These motifs are located 30 bp and 74 bp, respectively, upstream of the transcription start site. The sequence also revealed several E box (CANNTG) motifs between positions –896 and –629 relative to the transcription start site. Furthermore, a putative MEF2 binding site (GCTATATTTA) is located at position –860. When this upstream promoter region was attached to a reporter gene, it was found to drive tissue-specific expression in the skeletal muscle of carp (*Cyprinus carpio*) embryos as well as in the muscle of mature fish.

Accordingly the present invention provides an expression construct for expressing a polypeptide in fish, which construct comprises a nucleic acid coding

sequence encoding the polypeptide operably linked to a regulatory sequence capable of directing the expression of the coding sequence in a cell of the fish.

Preferably, the regulatory sequence is capable of directing expression of the coding sequence in, for example, a cell of a fish egg, a cell of an embryonic tissue, a cell of an extra-embryonic tissue, for example, in a cell of the yolk syncytial layer (YSL) and/or enveloping layer (EVL) of the fish egg. In another embodiment, the regulatory sequence is capable of directing expression of the coding sequence in a cell of the skeletal muscle.

Preferably, the polypeptides are pharmaceutical products with biological activity. These may be human factor VII, human factor VIII, human factor IX, calcitonin, an interleukin, an interferon, erythropoietin, tumour necrosis factor, α-galactosidase, adenosine deaminase, or any functionally active part of such a protein.

The present invention also provides a nucleic acid vector which comprises an expression construct of the invention.

The invention further provides a method for producing a transgenic fish, zygote, egg or embryo which method comprises introducing episomally an expression construct of the invention into a cell of the fish, zygote, egg or embryo. In another embodiment, the method of the invention comprises chromosomally incorporating a construct of the invention into the genome of the fish, zygote, egg or embryo.

The fish species involved will be of taxa *Cyprinidae*, *Cichlidae*, *Salmonidae*, *Claridae*, *Silumidae* or *Ictaluridae*, and will include zebrafish, (*Danio rerio*), African catfish (*Clarias gariepinus*), rainbow trout (*Orcorhynchus mykiss*), carp (*Cyprinus carpio*), or tilapia (*Oreochromis niloticus*).

The invention also provides a method for of producing a recombinant polypeptide, which method comprises expressing the polypeptide encoded by the coding sequence of the construct of the invention in a fish, fish egg or embryo and recovering the polypeptide

Preferably, the expression construct is introduced by microinjection. Alternatively it may be introduced by electroporation or lipofection.

Preferably the regulatory sequences comprise promoters giving ubiquitous or tissue specific expression, and include such regulatory sequences, introns, enhancers, polyA elements and other nucleic acid sequences as are necessary to maximise

expression in some or all tissues of fish eggs, embryos or adults. The regulatory sequences, in particular promoter sequences, used in the expression constructs are of viral or animal origin, are capable of driving expression in fish cells.

The expression of the coding sequences will be either transient, in which case the recovery will be from eggs or embryos, or stable, in which case the recovery will be from embryos or young fish, or mature fish.

Expression of polypeptides may be achieved by introducing the expression construct of the invention directly into somatic tissues of the adult fish preferably muscle tissue/cells. The gene constructs may also be introduced into germ line tissue/cells including gametes to achieve transmission to progeny fish.

Fish which have been made transgenic and which express the transgene copy or copies in a stable and chromosomally integrated manner may also be used for breeding so that eggs, embryos and other generations of expressing cells and fish can be produced.

Detailed Description of the Invention

It is possible in fish to express proteins, typically heterologous proteins, having pharmaceutical or biological properties. This technique allows the production of properly glycosylated and folded proteins, thus avoiding the disadvantages of microbial expression systems but is relatively inexpensive compared to the use of mammalian cell cultures or large mammalian hosts which, albeit for different reasons, both require a labour-intensive approach.

In particular, two general approaches are possible.

(i) Using a regulatory sequence capable of directing expression in fish muscle, or in another tissue, nucleic acid constructs incorporating the regulatory sequence and a coding sequence for a polypeptide of interest can be introduced into fish at a suitable stage in their development. The protein is then recovered from the muscle, and/or from other tissues, by harvesting the fish. For this approach, muscle-specific promoters, especially the myosin heavy chain (MyoHC) promoter, are preferred.

(ii) Polypeptides of interest can be expressed in and recovered from fish eggs, which eggs may be fertilised or unfertilised; or from developing zygotes or embryos. For example, a transgenic sexually mature fish can be produced which expresses the polypeptide of interest, under the control of a suitable the regulatory sequence, in her eggs. These eggs can then be removed and the protein extracted. This system has the advantage that no mature transgenic fish need be sacrificed to obtain the recombinant polypeptide. For this approach, a regulatory sequence capable of directing expression in a fish egg or developing zygote or embryo is required.

The polypeptides of the invention are produced by expressing DNA encoding them using an expression construct of the invention which comprises a nucleic acid coding sequence encoding the polypeptide operably linked to a regulatory sequence capable of directing the expression of the coding sequence in a cell of the fish.

The nucleic acid coding sequence may be DNA or RNA, preferably DNA, typically genomic DNA or cDNA. It may encode a polypeptide of any suitable length.

Any suitable polypeptide can be produced according to the invention, by expressing a nucleic acid, i.e. DNA or RNA, encoding it. Typically, such a polypeptide will be heterologous with respect to the fish system, i.e. it will be a polypeptide not naturally produced by the fish. However, it may be a homologous polypeptide.

Typically, the polypeptides produced in this way will have pharmaceutical and/or biological properties. Preferably the polypeptides of the invention are suitable for pharmaceutical use in mammals, more preferably humans. For example, they may be useful in treating, preventing or ameliorating a disease or other pathological condition of a human or animal subject.

Preferred polypeptides that may be produced in this way include blood clotting factors such as factors VII, VIII and IX; calcitonin; interleukins; interferons such as α-, β- and γ- interferon; α-galactosidase; tumour necrosis factor (TNF); and adenosine deaminase.

Preferably, such polypeptides will be produced in their complete (native) form, although the nucleic acid sequences of the invention may also encode functionally active parts of these polypeptides, for example parts of such polypeptides that have

the activity, or some of the activity, of the complete polypeptide. Similarly, the nucleic acid sequences of the invention may encode derivatives, for example mutated versions of the native polypeptides. For example, they may encode versions of the polypeptide that differ at one or more amino acids from the native version. Alternatively, the nucleic acid sequences may differ from the native sequence but encode the same amino acid sequence. Thus, the nucleic acid sequences may be degenerate of the native sequences. In this respect, the DNA sequences may be altered in such a way as to include codons that are preferred for expressing particular amino acids in fish cells; i.e. codons that are less efficiently expressed in fish may be replaced by more efficiently expressed ones.

In the constructs of the invention, the regulatory sequence of the invention is operably linked to a regulatory sequence capable of directing its expression in a cell of a fish. "Operably linked" refers to a juxtaposition wherein the regulatory sequence and the nucleic acid sequence encoding the polypeptide of the invention activity are in a relationship permitting the coding sequence to be expressed under the control of the promoter. Thus, there may be elements such as 5' non-coding sequence between the regulatory sequence and coding sequence, as long as they enhance, or do not impair, the correct control of the expression of the coding sequence by the regulatory sequence.

A regulatory sequence comprises at least a promoter sequence. In addition, a regulatory sequence may comprise other elements such as some or all of the following: a regulator of the promoter; an enhancer for the promoter; and/or a translational start site. The expression construct may also comprise a transcriptional terminator 3' to the sequence encoding the polypeptide of the invention. The expression construct may also comprise one or more introns or other non-coding sequences, for example, 3' or 5' to the sequence encoding the polypeptide of the invention. Such sequences can be included in the construct if they enhance or do not impair the correct control of the coding sequence by the promoter. The construct may also comprise a polyadenylation (polyA) signal operably linked 3' to the nucleic acid coding sequence, for example the SV40 polyA signal or the Chinook Salmon polyA signal. The constructs of the invention may also optionally comprise one or more Locus Control Regions (LCRs). These act as enhancers for certain fish genes and can

improve transgene expression (Aronrow *et al.* (1995) Mol. Cell. Biol. Vol 15; 1123-1135).

The constructs of the invention may be included within a vector, suitably a replicable vector, for instance a replicable expression vector.

In addition to the expression construct of the invention, a vector of the invention typically comprises an origin of replication so that the vector can be replicated in a host cell such as a bacterial host cell or a yeast host cell. A vector may also comprise additional elements involved in the control of expression of the coding sequence as discussed above.

The vector may also contain one or more selectable marker genes, typically an antibiotic resistance gene, for example an ampicillin resistance gene for the identification of bacterial transformants; or a marker gene that allows selection of yeast transformants. Optionally, the vector may also comprise an enhancer for the promoter. The expression vector may be of any type. The vector may be in linear or circular form. For example, the construct may be incorporated into a plasmid vector or a viral vector. Those of skill in the art will be able to prepare suitable vectors and constructs of the invention starting with widely available vectors which will be modified by genetic engineering techniques such as those described by Sambrook *et al.* (Molecular Cloning: A Laboratory Manual; 1989, Coldspring Harbor Press, NY, USA).

Any suitable regulatory sequence may be used in the constructs of the invention, as long as the regulatory sequence is capable of directing expression in a cell of a fish.

Regulatory sequences in the expression construct of the invention may be capable of directing expression in cells of some or all of the tissues of a fish. For example, they may direct expression wholly or mainly in certain tissues. Thus, they may be tissue-specific. For example, they may direct expression wholly or mainly in one or more particular fish tissues. Regulatory sequences that are specific for tissues of the fish egg or skeletal muscle are preferred.

It is preferred that the regulatory sequence directs expression in a cell of a fish egg such as a cell of an embryonic or extra-embryonic tissue of a fish egg. For example, the promoter may direct expression in a cell of an extra-embryonic tissue

such as a cell of the yolk syncytial layer (YSL) or enveloping layer (EVL) of the egg.

It is also preferred to use promoters capable of directing expression of the coding sequence in a cell of the muscle, typically the skeletal muscle, of a fish.

Regulatory sequences in the construct of the invention may be constitutive or inducible.

Specifically, one class of preferred regulatory sequences are the myosin heavy chain gene promoters. In particular, the carp FG2 myosin heavy chain promoter described by Gauvry *et al.* (1996), *ibid,* is preferred. This is a muscle-specific promoter, in the sense that it directs expression in cells of the skeletal muscle. Unexpectedly, it also directs expression in the YSL and EVL. A particularly preferred region of the FG2 MyoHC promoter is from the transcription start site to 901 bp upstream. This type of tissue-specific regulatory sequence is particularly preferred when the expression construct is to be stably introduced into the fish genome long-term, especially into the genome of germline cells. More generally, fish-derived tissue-specific regulatory sequences are preferred where long-term expression and/or stable integration of the expression construct into the fish cell genome, rather than transient expression is required. It is also preferred to use promoters which are inducible such that expression of the polypeptide can be induced by a suitable stimulus at the desired time. This may avoid any side-effects associated with the continuous expression of a heterologous polypeptide.

Other preferred regulatory sequences include the cytomegalovirus (CMV) promoters; (e.g. the CMV 1E1 promoter) the Friend murine leukaemia virus long terminal repeat promoter (MuLV-LTR); the thymidine kinase, (TK) promoter, optionally in combination with the Rous Sarcoma Virus (RSV) enhancer; the mouse metallothionein I (mMTHI) promoter; the *Xenopus* elongation factor 1α (eflα) promoter, optionally in combination with the eflα enhancer; the carp β-actin promoter; the mouse Hox 1.3 promoter; the rat GAP 43 gene promoter; the SV40 early promoter; the rabbit β-cardiac myosin heavy chain promoter; and the human MxA promoter. Particularly preferred for transient expression are ubiquitous and/or constitutive regulatory sequences allowing high level expression in all cell types. Viral regulatory sequences, for example CMV and RSV sequences are especially preferred.

Optionally, an enhancer may also be used in addition to the promoter. The enhancer will be chosen to be compatible with the promoter. Some suitable enhancers include the rat foetal light chain enhancer, the ef1α enhancer and the RSV enhancer.

Any suitable type of fish may be used, according to the invention, for the production of polypeptides of interest. Teleost fish are preferred. Amongst teleost fish, preferred taxa include *Cyprinidae*, *Cichlidae*, *Salmonidae*, *Claridae*, *Siluridae* and *Ictaluridae*. Some particularly preferred types of fish are carp (e.g. *Cyprinus carpio*), zebrafish (*Danio rerio*), African catfish (*Clarias gariepinus*), tilapia (*Oreochronis niloticus*) atlantic salmon (*Salmo Salar*), rainbow trout (*Oncorhyncus mykiss*), medaka (*Oryzias latipes*). Generally, when it is desired to recover polypeptides from fish eggs, or from developing zygotes or embryos preferred fish for use according to the invention are able to produce large eggs and/or large numbers of eggs. Also, it is desirable to use fish for which propagation and/or transgenic technology is well developed.

The present invention also provides cells harbouring the vectors or constructs of the invention. These cells may be of any type. For example, they may be microorganism cells. Thus, they may for example be bacterial, e.g. *E. coli*, or yeast cells.

Preferred cells of the invention are fish cells. Cells of any type of fish are within the scope of the invention, and cells of the fish taxa mentioned herein are preferred. Similarly, the cells may be cells of any type of fish tissue, although tissues mentioned herein are preferred. Cells of the fish egg, for example cells of embryonic tissues, or of extra-embryonic tissues such as the YSL and EVL, are particularly preferred. Muscle cells, typically skeletal muscle cells are also preferred.

Fish cells of the invention may be in any form. They may be isolated, e.g. in culture, or in a fish, e.g. a mature fish; or in a fish zygote, embryo or egg. Thus, the invention provides transgenic fish comprising cells of the invention. These may be at any stage of development. Thus, for example, the invention provides mature and immature transgenic fish, as well as transgenic fish zygotes, embryos and eggs. In such fish, some or all of the cells harbour constructs or vectors of the invention. Thus, the fish may be uniformly transgenic or transgenic "mosaics" in which only some cells are transgenic. Cells of the invention may be localised in particular tissue types,

for example in one or more tissues such as the skeletal muscle, germline cells or eggs.

The transgenic fish of the invention may be of either sex; i.e. they may be male or female.

The constructs and vectors of the invention may be introduced into cells, to make cells of the invention, by any suitable means (for example, as set out in Sambrook *et al.* (1989) *ibid*). For example, the cells may be transformed or transfected by any means known in the art. For example, constructs and vectors of the invention may be packaged into infectious viral particles, for example retroviral, particles or lambda virus particles which are then used to transfer the constructs or vectors to the cells. Also, they may be introduced by microinjection, electroporation, calcium phosphate precipitation, biolistic methods, (e.g. tungsten bombardment) or by contacting naked nucleic acid vectors or constructs with the cells in solution. Optionally, agents that facilitate DNA transfer into cells may be used. These include liposomes. When DNA or RNA is introduced into fish eggs, a preferred means of delivery is microinjection, using techniques known to a person of skill in the art. Microinjection can be performed at any suitable stage in the development of the egg, for example prior to fertilisation; or at the one-cell, two-cell, four-cell or eight-cell stage. Another preferred technique for delivering nucleic acids to fish eggs, as well as to fish sperm, is electroporation which is reviewed extensively in Transgenic Animals – Generation and Use (1997) pp 129-132, ed. L. M. Houdebin, Harwood Acedemic Publishers.

The vectors or constructs introduced into cells may be of any suitable type. For example, they may be able to integrate constructs of the invention into the cell genome or they may be episomal, and remain free in the cell.

The invention also provides methods of making transgenic fish of the invention. Any suitable method may be used. In principle, the methods of the invention involve introducing a construct or vector of the invention into a cell, generating a cell of the invention as described above. If the cell is *in vivo*, for example in a fish, or fish zygote embryo or egg, this leads to a transgenic fish, zygote, embryo or egg of the invention. If appropriate, the fish can be reproduced to produce transgenic progeny fish of the invention. Fish eggs of the invention can be fertilised as appropriate and then grown up to yield fish of the invention, which may then be

reproduced to yield further progeny fish of the invention. Fertilised fish eggs, zygotes and embryos of the invention may be grown up to yield fish of the invention which may then be reproduced to yield further progeny fish of the invention. Progeny fish of the invention may also be reproduced to generate further fish of the invention.

Also, fish sperm can be transformed with constructs of the invention and this transgenic sperm can be used to generate transgenic fish of the invention by fertilising fish eggs, optionally transgenic eggs of the invention.

Typically, cells or fish (including eggs, zygotes and embryos) are subjected to suitable selection procedures to separate transformed cells or fish from non-transformed ones.

It is also possible to introduce vectors or constructs of the invention into fish cells in culture, and then incorporate the cells into the fish, for example into mature or immature fish or zygotes, eggs or embryos, to generate fish of the invention. These are optionally reproduced or grown up as appropriate to generate fish of the invention.

Preferably, in the methods of the invention, the construct of the invention, or a part of the construct is chromosomally incorporated into the fish cell genome, for example using an integrative vector. Where a part of the construct is integrated, it is preferably a part comprising the coding sequence, more preferably a part comprising the coding sequence and flanking sequences (e.g. the promoter) capable of directing its expression in the fish cell. One or more copies of the coding sequence may be integrated into each cell.

The vector or construct of the invention may be introduced into the fish cell at any suitable stage in the fish life cycle. For example, it may be introduced into an unfertilised egg, or a fertilised zygote, or a developing embryo, or into an immature fish or a mature fish. Thus obtained unfertilised eggs may be fertilised to yield zygotes of the invention, which may then be grown up to yield embryos and then fish of the invention. Female fish of the invention may produce transgenic eggs of the invention which can be fertilised, optionally by transgenic male fish of the invention, or sperm derived from such fish.

The invention also provides methods for producing recombinant polypeptides of the invention by expressing the polypeptide encoded by the coding sequences of the invention in a cell of the invention, and recovering the polypeptide thus produced

by any means known in the art.

Polypeptides of the invention may be produced and recovered from cells of the invention cultured *in vitro*. However, it is preferred to produce polypeptides of the invention *in vivo* in the cells of living fish of the invention, for example cells of mature or immature fish, or their zygotes, eggs or embryos of the invention.

The polypeptide may be obtained by harvesting mature fish, or fish eggs, embryos and zygotes and recovering the polypeptide by any suitable means. Optionally, the polypeptide may be isolated, or substantially isolated, e.g. by one or more isolation steps. Optionally, the polypeptide may be purified, completely or partially, e.g. by one or more purification steps.

In a preferred embodiment, mature female transgenic fish of the invention are produced and allowed to produce eggs which eggs may optionally be fertilised. The eggs are harvested and the polypeptide of the invention is then recovered from the eggs. In methods of this nature, the female fish may be superovulated to enhance the number of eggs produced, and thus the amount of polypeptide recovered. Optionally, in such methods, expression is specifically obtained in the eggs by means of a suitable promoter.

In another preferred embodiment, mature transgenic fish of the invention are generated, and polypeptides are expressed in their tissues. Preferably, expression takes place selectively, in the skeletal muscle, e.g. under the control of a muscle-specific promoter such as the carp FG2 MyoHC promoter described in Gauvry *et al* (1996), *ibid.*

In another embodiment, a DNA or RNA sequence encoding a polypeptide of the invention can be used to achieve transient expression of the polypeptide in a developing fish egg, zygote or embryo. RNA encoding the polypeptide is introduced into a fish egg, optionally a fertilised egg. The egg is, if appropriate, fertilised and grown up to a suitable stage in development, which allows the polypeptide to be expressed.

The polypeptide expressed is recovered by any means known in the art. The polypeptide may be recovered from the eggs at any stage in their development.

The invention will be described with reference to the following Example which are intended to be illustrative only and not limiting. The Example refers to the

Figures. Referring to the Figures in more detail:

Figure 1 is the Invitrogen™ expression vector into which the FVII cDNA is inserted.

Figure 2 is the construct from which the FVII cDNA was isolated.

Figure 3 is the construct formed by inserting the FVII cDNA into the expression vector in Figure 1.

Figure 4 is a graph showing catfish embryos expressing recombinant human factor VII.

EXAMPLE

PRODUCTION OF RECOMBINANT HUMAN BLOOD CLOTTING FACTOR-VII IN FISH EMBRYOS

MATERIALS AND METHODS

Plasmids

The DNA encoding human factor VII has been cloned and sequenced (O'Hara et al. (1987) PNAS, Vol 84; 5158-5162 – Genbank Accession No. J02933). The particular human factor VII coding gene cDNA used in these experiments was isolated by HindIII/BamHI double digestion from the plasmid containing the FVII sequence flanked by a 0.9 kb rat myosin heavy chain gene-derived muscle specific regulatory element (pMyHC/FVII). (Examples of rat myosin heavy chain promoter sequences are published as Genbank Accession Nos. U83321 and U55179). The 2.62 kb FVII cDNA fragment containing the 2.46 kb FVII cDNA and the 0.12 kb SV40 polyA signal sequences was ligated into the pcDNAI expression vector (Invitrogen™) into the HindIII/BamHI sites. The resulting plasmid pCMV/FVII (Fig 2) was checked for the appropriate insertion and orientation by restriction mapping. It contains the CMV virus E1 early promoter and enhancer 5' to the FVII sequences. The plasmid was prepared using conventional molecular techniques (Sambrook et *al.*, (1989), *ibid*) and used for microinjection into fertilised fish eggs.

Microinjection and embryo culture

Embryos of African catfish were produced by artificial propagation of African catfish broodstock The females were injected with 3 mg/kg body weight carp pituitary dissolved in PBS solution 8 hours prior to planned ovulation. The eggs were stripped of the females and fertilised by addition of sperm derived from testis surgically removed from males. Single cell embryos (zygotes) were used for microinjection.

Zebrafish embryos were gained from natural spawnings of broodstock kept at 14 hours light 10 hours dark cycle at 28°C. Eggs were late into spawning boxes (Westfield, M (1993) The zebrafish book: A guide for the laboratory use of zebrafish (Brachydanio rerio), University of Oregon Press, Eugene, OR, USA) and collected from the boxes for microinjection at the early developmental stages (1-2 cell stage).

The pCMV/FVII and the pMyHC/FVII plasmids were both microinjected in circular form into African catfish or zebrafish embryos at the zygote stage in approximately 10^6 to 10^7 copies using a pressure driven picoinjector. The embryos of catfish were placed on Petri dishes where the naturally sticky eggs remained attached. The injection was done by hand using glass microcapillary filled with the appropriate DNA solution containing blue food dye for localisation of injection drop. The zebrafish embryos were placed into an agar gel injection mould designed to hold eggs in row for injection (Westerfield et al, 1993). The injection in case of both species was targeted into the cytoplasmic region or the boundary between the cytoplasmic and yolk regions of the egg. The microinjected embryos were cultured for one day at 28°C in thermostat in Holtfreter's solution until reaching the prim 5 stage (prior to hatching). The microinjected and control (non injected) embryos were collected into microcentrifuge tubes homogenised by plastic homogeniser. The tubes containing up to 1 to 100 homogenised embryos (approx 20 µl) were snap frozen in liquid nitrogen and stored at -70°C until analysis.

RESULTS

Immunochemical detection of FVII protein from fish embryo homogenates

ELISA assay was carried out in 96 well plates coated with monoclonal

antibody raised against human FVII (murine monoclonal antibody Clone HVII-1, Sigma), Fish embryo homogenates, dilutions of normal blood plasma containing FVII protein and appropriate negative control samples were loaded into the wells. Biotinylated anti-FVII TAG Antibody conjugated with horse radish perodiase was then added. After addition and incubation with the colour reaction buffer, the samples were read in a conventional ELISA reader. Results of zebrafish and catfish embryos are shown in table I and Fig 4 respectively.

Activity Assay

Enzymatic activity of the recombinant factor VII produced in the fish embryos was measured by a chromogenic assay Coaset FVII (Chromogenix, Sweden). The assay is based on a two stage principle. In stage 1, human factor X is activated (FXa) by thromboplastin in the presence of Ca^{2+} and FVII in the extrinsic pathway. FVII is completely converted to FVIIa during this process so there is no pre-activated FVII Interference in the assay. In stage 2, the generated FXa hydrolyses the chromogenic substrate S-2765, liberating the chromophoric group pNA. The colour is read photometrically at 405 nm. The generated FXa thus increases the intensity of the colour which is therefore proportional to FVII activity in the sample. The assay was carried out in a microtitre plate which had been pre-coated with a 1% solution of BSA in PBS to prevent non-specific proteins binding during the assay. A standard curve was made from normal blood plasma (pooled from 20 individuals with no thrombotic or bleeding history) and used to prepare a standard curve in the range 200 U/dl to 12.5U/dl by doubling dilutions from 1:5000. Plasma samples were diluted 1:1000.

Fish embryo samples were measured undiluted and in doubling dilutions from 1:40. The data shown in table II was taken from the 1:1280 dilutions of the samples. Results were multiplied by 1.28 to match the normal plasma dilution of 1:1000, and expressed in ng/ml on the basis that 100 U/dl is equivalent to the accepted normal plasma concentration of 2000 ng/ml. Results of activity assay in zebrafish embryos are shown in table II.

Blood clotting assay

Factor VII deficient plasma was used for blood clotting assays (Chanarian, I.

in Laboratory Haematology, Churchhill Livingston, London 1989 pages 286-287) derived from immuno depleted plasma (Diagnostic Reagents Ltd). Thromboplastin and the appropriate samples were added followed by the addition of $CaCl_2$. The time lapse of the clotting reaction was measured in a Coagulometer KC10. The time lapse results were compared to dilutions of normal plasma samples using a logarithmic scale and were expressed as the percentage activity of normal plasma, where normal plasma means 100 U/dl FVII protein. The results of the blood clotting assay are shown in table III.

Table 1

ELISA test on zebrafish embryos for detection of human FVII protein

	Number of embryos (db)	**FVII concentration (ng/ml)**
embryos microinjected with pMyHC/FVII:	100	85
	100	32
embryos microinjected with pCMV/FVII:	100	186
	100	246
	119	421
control embryos	100	30
FVII dilution series:	**FVII from serum applied (ng/ml)**	**FVII concentration measured (ng/ml)**
	500	508
	250	239
	125	109
	63	65
	16	14
	0	8

Table II

Activity assay for recombinant FVII produced in fish embryos:

	Number of embryos (db)	FVII concentration (ng/ml)
embryos microinjected with pCMV/FVII:	14	80
	100	880
	50	540
non injected control embryos	100	0

Table III

Results of bioassay for the blood clotting activity of recombinant FVII produced in fish embryos using FVII deficient blood plasma

	Number of	% activity *
pCMVFVII injected embryos		
1.	100	21
2.	100	25
3.	100	28
4.	100	22
5.	50	29
non injected control embryos		
1.	100	8
2.	10	8
control blood plasma		100
bank		8

* Blood clotting activity is measured in the % of the activity of normal human blood plasma containing normal levels of FVII (100 u/dl).

CLAIMS

1. An expression construct for expressing a polypeptide in fish, which construct comprises a nucleic acid coding sequence encoding the polypeptide operably linked to a regulatory sequence capable of directing the expression of the coding sequence in a cell of the fish.

2. A construct according to claim 1 wherein the regulatory sequence is capable of directing expression of the coding sequence in a cell of a fish egg.

3. A construct according to claim 2 wherein the cell is a cell of an extra-embryonic tissue.

4. A construct according to claim 3 wherein the regulatory sequence is capable of directing expression in a cell of the yolk syncytial layer (YSL) and/or enveloping layer (EVL) of the fish egg.

5. A construct according to claim 2 wherein the cell is a cell of an embryonic tissue.

6. A construct according to claim 1 or 2 wherein the regulatory sequence is capable of directing expression of the coding sequence in a cell of the skeletal muscle.

7. A construct according to any one of the preceding claims wherein the coding sequence encodes a polypeptide selected from Factor VII, Factor VIII, Factor IX, calcitonin, an interleukin, an interferon, erythropoietin, tumour necrosis factor, α-galactosidase and adenosine deaminase; or a functionally active portion thereof.

8. A construct according to claim 7 wherein the coding sequence encodes Factor VII.

9. A construct according to any one of the preceding claims wherein the

regulatory sequence comprises the cytomegalovirus (CMV) promoter.

10. A construct according to any one of the preceding claims wherein the regulatory sequence is capable of directing expression in a cell of a teleost fish.

11. A construct according to any one of the preceding claims wherein the fish is of the taxon *Cyprinidae*, *Cichlidae*, *Salmonidae*, *Claridae*, *Siluridae* or *Ictaluridae*.

12. A construct according to claim 11 wherein the fish is a zebrafish, African catfish, trout, carp or tilapia.

13. A nucleic acid vector which comprises a construct as defined in any one of the preceding claims.

14. A cell harbouring a construct as defined in any one of the preceding claims.

15. A cell according to claim 14 wherein the construct, or a part thereof, is chromosomally incorporated.

16. A cell according to claim 15 wherein the construct is not chromosomally incorporated.

17. A cell according to any one of claims 14 to 16 which is a fish cell.

18. A cell according to claim 17 which is the cell of a fish as defined in any one of claims 10 to 12.

19. A cell according to claim 17 or 18 which is a cell as defined in any one of claims 2 to 6.

20. A transgenic fish, fish zygote, fish egg or fish embryo comprising a cell according to any one of claims 14 to 19.

21. A method for producing a transgenic fish, fish zygote, fish egg or embryo, as defined in claim 20, which method comprises chromosomally incorporating a construct as defined in any one of claims 1 to 12, or a vector as defined in claim 13; or a part thereof comprising the coding sequence; into the genome of the fish, zygote, egg or embryo.

22. A method for producing a transgenic fish, zygote, egg or embryo as defined in claim 20 which method comprises introducing episomally a construct as defined in any one of claims 1 to 12 or a vector as defined in claim 13 into a cell of the fish, zygote, egg or embryo.

23. A method according to claim 21 or 22 wherein the construct is introduced into a cell of the skeletal muscle of a fish or fish embryo.

24. A method according to claim 21 or 22 wherein the construct is introduced into a fish egg or a fish zygote.

25. A method according to claim 21 or 22 wherein the construct or vector is introduced into a cell in culture, and the resultant cell is incorporated into a fish, or fish egg, zygote or embryo.

26. A method of producing a recombinant polypeptide, which method comprises expressing the polypeptide encoded by the coding sequence of the construct of any one of claims 1 to 12 or the vector of claim 13 in a cell according to any one of claims 14 to 19, and recovering the polypeptide produced.

27. A method according to claim 18, which method comprises culturing *in vitro* a cell as defined in any one of claims 14 to 19 and recovering the polypeptide produced.

28. A method according to claim 27, which method comprises expressing the polypeptide in a fish, fish zygote, fish egg or fish embryo according to claim 20 or

produced by the method of any one of claims 21 to 24, and recovering the polypeptide.

29. A method according to claim 27, which method comprises producing a mature transgenic fish by the method of claim 21, 22, or 25 allowing the fish to produce eggs which express the polypeptide, optionally fertilising the eggs, and recovering the polypeptide from the eggs or from zygotes, embryos or fish derived therefrom.

30. A method for producing a recombinant polypeptide which comprises introducing an RNA molecule comprising an RNA sequence encoding said polypeptide into a fish egg under conditions that allow the polypeptide to be expressed; and recovering the polypeptide thus produced.

31. A method according to claim 30 wherein the polypeptide is recovered from the eggs.

32. A method according to claim 31 wherein the eggs are allowed to develop into embryos and the polypeptide is recovered from the embryos.

33. A method according to claim 30 wherein the eggs are eggs of fish as defined in claim 10 or 11.

Fig.1.

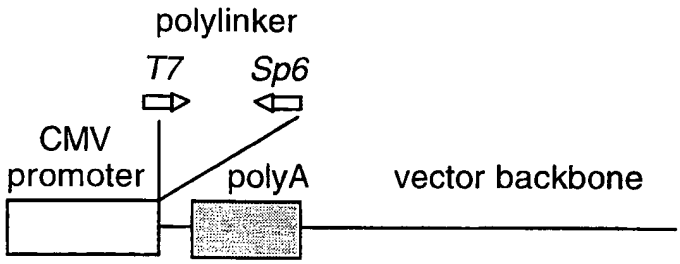


Fig.2.

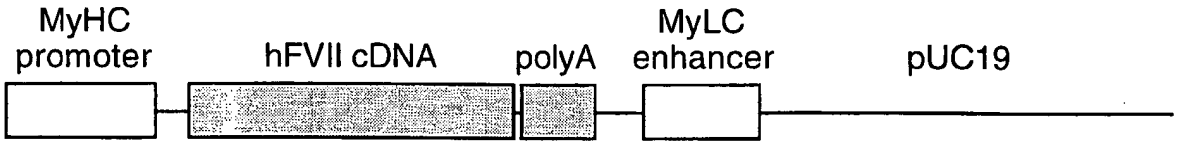


Fig.3.

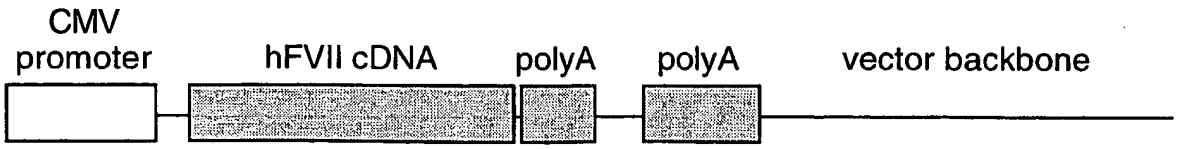
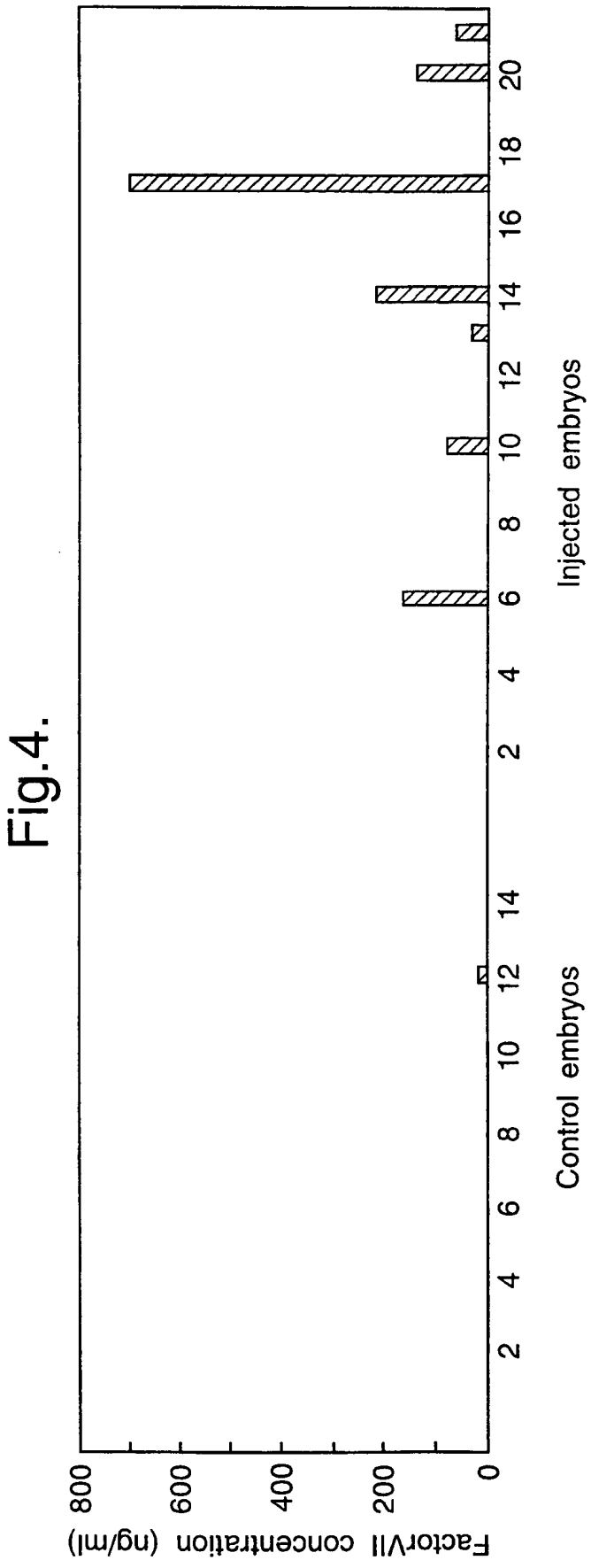


Fig.4.


FactorVII concentration (ng/ml)
800
600
400
200
0
2
4
6
8
10
12
14
Control embryos
2
4
6
8
10
12
14
16
18
20
Injected embryos

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 97/02806

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A01K67/027 C07K14/745

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUELLER, F. ET AL.: "The use of transient LacZ expression in fish embryos for comparative analysis of cloned regulatory elements" SOCIETY FOR EXPERIMENTAL BIOLOGY SEMINAR SERIES : GENE EXPRESSION AND MANIPULATION IN AQUATIC ORGANISMS, vol. 00, no. 58, June 1996, CAMBRIDGE, UK, pages 175-199, XP002056598 see the whole document --- -/--	1-3,5,6, 10-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1998	06/03/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MÜLLER, F. ET AL.: "Efficient transient expression system based on square pulse electroporation anmd in vivo luciferase assay of fertilized fish eggs" FEBS LETTERS, vol. 324, no. 1, June 1993, AMSTERDAM NL, pages 27-32, XP002056599 see page 31, column 2, paragraph 5 - page 32, column 1, paragraph 2; figure 5B	1-3,5,6, 9-24
X	GONG Z ET AL: "FUNCTIONAL ANALYSIS AND TEMPORAL EXPRESSION OF PROMOTeR REGIONS FROM FISH ANTIFREEZE PROTEIN GENES IN TRANSGENIC JAPANESE MEDAKA EMBRYOS" MOLECULAR MARINE BIOLOGY AND BIOTECHNOLOGY, vol. 1, no. 1, 1 September 1991, pages 64-72, XP000283150 see the whole document	1-5, 10-24
X	ZHU Z ET AL: "NOVEL GENE TRANSFER INTO THE FERTILIZED EGGS OF GOLD FISH (CARASSIUS AURATUS L. 1758)" JOURNAL OF APPLIED ICHTHYOLOGY - ZEITSCHRIFT FUER ANGEWANDTE ICHTHYOLOGIE, no. 1, 1985, pages 31-34, XP000675982 see the whole document	1,2,5, 10-24
X	WO 92 16618 A (HSC RES DEV LP ;SEABRIGHT CORP LTD (CA)) 1 October 1992 see the whole document	1,10-24
X	GAUVRY, L. ET AL.: "The characterisation of the 5' regulatory region of a temperature-induced myosin heavy-chain gene associated with myotomal muscle growth in the carp" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, no. 3, 2 March 1996, pages 887-894, XP002056600 cited in the application see the whole document	1,6, 10-19
X	ZHANJIANG LIU ET AL: "DEVELOPMENT OF EXPRESSION VECTORS FOR TRANSGENIC FISH" BIO/TECHNOLOGY, vol. 8, no. 12, 1 December 1990, pages 1268-1272, XP000293044 see the whole document	1,2, 10-20

-/--

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 97/02806

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIN, S. ET AL.: "Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 89, May 1992, WASHINGTON US, pages 4519-4523, XP002056601 see the whole document	1,2,5, 10-25
P,X	WILLIAMS, D.W. ET AL.: "High transgene activity in the yolk syncytial layer affects quentitative transient expression assays in zebrafish (Danio rerio) embryos" TRANSGENIC RESEARCH, vol. 5, no. 6, November 1996, pages 433-442, XP002056602 see the whole document	1-5, 10-24
P,X	MÜLLER, F. ET AL.: "Activator effect of coinjected enhancers on the muscle-specific expression of promoters in zebrafish embryos" MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 47, no. 4, August 1997, pages 404-412, XP002056603 see the whole document	1-6, 10-24
P,X	WO 96 32087 A (UNIV DALHOUSIE ;WRIGHT JAMES R JR (CA); POHAJDAK BILL (CA)) 17 October 1996 see the whole document	1,2,5, 10-24

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/GB 97/02806

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9216618 A	01-10-92	AU 669844 B	27-06-96
		AU 1370392 A	21-10-92
		CA 2106315 A	16-09-92
		EP 0578653 A	19-01-94
		JP 6505870 T	07-07-94
		NO 933276 A	11-11-93
		US 5545808 A	13-08-96
WO 9632087 A	17-10-96	AU 4935596 A	30-10-96
		CA 2191969 A	17-10-96
		EP 0769021 A	23-04-97
		NO 965157 A	03-12-96